

SUPPLEMENTAL DIGITAL CONTENT

Exposure Of Stored Packed Erythrocytes To Nitric Oxide Prevents Transfusion–Associated Pulmonary Hypertension

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**Deceased*

Supplemental Methods:

Animal preparation and hemodynamic monitoring

Seven groups of awake lambs were studied and randomized to the different study groups. One group of lambs (n=4) received control-treated fresh packed erythrocytes, a second group (n=8) was transfused with nitric oxide gas-treated fresh packed erythrocytes, in a third group (n=8) transfusion was performed with untreated stored packed erythrocytes and a fourth group (n=9) received nitric oxide gas-treated stored packed erythrocytes. Two additional

groups were transfused with either fresh (n=4) or stored (n=6) MAHMA NONOate-treated packed erythrocytes whereas a seventh group (n=5) received washed stored packed erythrocytes. The researcher who performed the hemodynamic measurements and analyses was blinded to the group and treatment assignment.

Animals were anesthetized with 2-3% isoflurane (Piramal Critical Care, Inc., Bethlehem, PA) in oxygen via a mask. After endotracheal intubation of the lamb, an 18 G catheter was placed in the right carotid artery. A 7 FR Swan-Ganz catheter was inserted in the pulmonary artery using a 8.5 Fr sheath introducer set with an integral hemostasis port (ID 2.8 mm, placed in the right jugular vein) that was used for the blood transfusion. Cefazolin (10 mg/kg) was given intravenously for perioperative antibiotic prophylaxis. After surgery, animals were extubated and allowed to recover from anesthesia in a Babraham metabolic cage for 2 h¹.

The lambs were gently restrained to prevent them from inadvertently removing invasive catheters, but the restraints allowed the animals to stand up or sit at will. On rare occasions, if the lambs appeared to be distressed, the animals were on rare occasion sedated with an IV bolus of 0.01-0.02 mg/kg midazolam.

Mathers and colleagues demonstrated in dogs that 1.0 minimal alveolar concentration (MAC) of isoflurane is needed to lower PAP and 2.0 MAC to lower pulmonary vascular resistance². Our lambs were extubated after surgery when levels of isoflurane were below 0.17 MAC. We believe that, after 2 h of air breathing during the recovery period, there were insufficient isoflurane levels

remaining to influence the pulmonary vasomotor response to transfusion in our awake lambs.

Hemodynamic parameters, including mean systemic arterial blood pressure (MAP), heart rate (HR), central venous pressure (CVP), and mean pulmonary arterial pressure (PAP) were monitored continuously and the pulmonary capillary wedge pressure (PCWP) was measured intermittently every 10-30 min for 4 h after beginning packed erythrocytes transfusion. Cardiac output was assessed by thermodilution as the average of three measurements after intravenous bolus injection of 10 ml of 0°C saline. Systemic vascular resistance index (SVRI), pulmonary vascular resistance index (PVRI) and cardiac index were calculated using standard formulae.

After all hemodynamic measurements were completed, lambs were anesthetized with 2% isoflurane in oxygen, all catheters were removed, and the right carotid artery was ligated. After recovery from anesthesia, sheep were housed and blood was sampled by venipuncture in the animal facility on days 1 and 7 to determine the lifespan of transfused erythrocytes.

Nitric oxide consumption assay

The nitric oxide consumption assay³ is an *in vitro* test to measure the amount of nitric oxide that can be scavenged by a solution containing cell-free hemoglobin and microvesicles containing hemoglobin. Briefly, 0.01mM DETA NONOate, a nitric oxide donor with a half-life of 56 h at 25°C, was equilibrated in an anaerobic purge vessel flushed with helium gas entering a nitric oxide

chemiluminescence analyzer (Sievers, Boulder CO). When the nitric oxide concentration achieved a steady state (after 20-30min), samples of supernatant from the stored blood units are injected into the solution and the instantaneous decrease in nitric oxide concentration is quantified.

Experimental assay of deformability of nitric oxide-treated packed erythrocytes using microfluidics

Blood samples (1.5 ml) were obtained from fresh and stored ovine packed erythrocytes units after transient *ex vivo* exposure to 300 ppm NO in 90% N₂/10% O₂ or a control gas mixture (90% N₂/10% O₂). One microliter of the packed erythrocytes sample was pelleted and re-suspended in 200 µl PBS solution containing 1% w/v bovine serum albumin (BSA). The addition of BSA prevented cell adhesion to walls of the container, while the low cell density prolonged the time that cells remained in a single-cell suspension.

The microfluidic device is an optimized design of the device described by Bow and co-workers⁴ to study the deformability of individual erythrocytes. Standard microfabrication and soft lithography were used to fabricate polydimethylsiloxane (PDMS) microfluidic devices as described⁴. In brief, silicon wafers with the desired pattern were created using SU-8 photoresist and UV exposure. We then silanized wafers using trichloro(1H,1H,2H,2H-perfluorooctyl)silane (FOTS). PDMS prepolymer (Sylgard 194, Dow Corning, Midland, MI) was mixed with curing agent in a ratio of 10:1 and then cast on

patterned wafers with 2 h of curing at 80°C. Inlets and outlets were created in the PDMS device using a 1.5mm biopsy punch. The patterned PDMS then was bonded to a glass slide using oxygen plasma treatment via an RF source (Harrick Plasma).

The erythrocytes flowed through a large microchannel (1mm wide and 5mm long) facing three periodic sets of rectangular capillaries (Figure 10). The cross section of capillaries or “slits” were 5 μm high and 2 μm wide with an inverted trapezoidal shaped flow entrance. The trajectories of individual erythrocytes were monitored through an inverted microscope (Zeiss Axiovert 200) equipped with a CCD camera (Hitachi KP-D20AU) at 30 frames per second and fed to a PC via a Labview interface. “Individual transit erythrocyte velocity” was defined as the velocity of the erythrocytes within each slit; averaged over three periodic slit sets. This velocity was obtained by post-imaging analysis ImageJ software (NIH, Bethesda, MD). Average erythrocyte velocity for each sample was computed from 134 individual erythrocyte transit velocities.

Supplemental Results

Treatment of stored packed erythrocytes with MAHMA NONOate reduces nitric oxide consumption by supernatant hemoglobin

Treatment of stored packed erythrocytes prior to transfusion with the nitric oxide-donor compound MAHMA NONOate was studied as an alternative approach to nitric oxide gas exposure of stored packed erythrocytes. We tested whether in vitro treatment of stored packed erythrocytes units with MAHMA NONOate would reduce scavenging of nitric oxide by hemoglobin in storage unit supernatant. Comparisons were performed on the change in cell-free hemoglobin, nitric oxide consumption, and met-hemoglobin in MAHMA NONOate-treated packed erythrocytes versus control-treated packed erythrocytes.

As previously noted, the hemoglobin concentration in the supernatant of stored packed erythrocytes was greater than that in the supernatant of fresh packed erythrocytes. Treatment of stored packed erythrocytes with MAHMA NONOate (at a final concentration of 200 μM) did not increase the level of supernatant hemoglobin, suggesting that MAHMA NONOate treatment did not induce hemolysis (Supplemental Figure 1A).

To investigate the nitric oxide-scavenging effects of supernatant hemoglobin exposed to MAHMA NONOate, we measured the nitric oxide-consumption of packed erythrocytes unit supernatant before and after incubation with the nitric oxide-donor. We found the fresh packed erythrocytes unit

supernatant scavenged far less nitric oxide than the stored packed erythrocytes unit supernatant (Supplemental Figure 1B). Incubation of stored packed erythrocytes supernatant with MAHMA NONOate reduced nitric oxide consumption as compared with untreated stored packed erythrocytes supernatant (Supplemental Figure 1B, reduction in nitric oxide-consumption by $81.2 \pm 14.8 \mu\text{M}$ vs. $7.4 \pm 10.2 \mu\text{M}$, $p=0.0007$).

To test whether the reduction in nitric oxide-consumption might be related to an increase in extracellular met-hemoglobin, we measured the extracellular fraction of met-hemoglobin by spectral deconvolution⁵. Pre-treatment with the nitric oxide-donor compound increased the percent of extracellular met-hemoglobin in packed erythrocytes storage supernatant from $1 \pm 0.4\%$ to $32 \pm 5\%$, $p=0.001$ (fresh blood) and from $5 \pm 3\%$ to $68 \pm 4\%$, $p<0.0001$ (stored blood) respectively (Supplemental Figure 1C).

The percentage of intracellular met-hemoglobin was measured to evaluate the erythrocyte oxygen transport capacity after incubation with MAHMA NONOate. Intracellular met-hemoglobin levels increased to $3 \pm 0.2\%$ when either fresh or stored packed erythrocytes were incubated with MAHMA NONOate for 20 min (Supplemental Figure 1D). The intracellular met-hemoglobin concentrations did not differ from baseline levels at 2 h after treatment and incubation with MAHMA NONOate (data not shown).

These findings demonstrate that the ability of the supernatant of stored packed erythrocytes units to consume nitric oxide was reduced after MAHMA NONOate treatment, via conversion of oxy-hemoglobin to met-hemoglobin. Treatment with

the nitric oxide-donor compound MAHMA NONOate did not cause hemolysis of packed erythrocytes. Furthermore, because the level of intracellular methemoglobin was increased to only 3% and rapidly returned to normal, the oxygen transport capacity of packed erythrocytes was not permanently impaired by MAHMA NONOate treatment.

Incubating stored packed erythrocytes with the nitric oxide-donor compound MAHMA NONOate prevents transfusion-associated pulmonary hypertension

Pulmonary hemodynamic parameters were measured in awake lambs before, during and after transfusion of one unit of fresh or stored MAHMA NONOate-treated packed erythrocytes. When stored packed erythrocytes were exposed to the nitric oxide-donor compound before transfusion, PAP did not increase when compared to fresh packed erythrocytes (Supplemental Figure 2A, PAP at 20 min of 14.5 ± 0.8 mmHg vs. 13.9 ± 0.6 mmHg, $p > 0.01$), indicating that treatment with MAHMA NONOate prevented the transfusion-related increase in PAP. Transfusion of fresh packed erythrocytes, whether treated with the nitric oxide-donor compound or not, did not change the PAP (Supplemental Figure 2A).

Pre-treatment of a stored packed erythrocytes unit with the nitric oxide-donor compound prevented the increase in PVRI (Supplemental Figure 2B, PVRI at 20 min of 118.5 ± 12 dyn \cdot sec \cdot cm $^{-5}$ \cdot m $^{-2}$ vs. 107.5 ± 17.8 dyn \cdot sec \cdot cm $^{-5}$ \cdot m $^{-2}$, $p > 0.05$). Transfusion of a fresh packed erythrocytes unit with or without adding MAHMA

NONOate did not alter PVRI (Supplemental Figure 2B). These results show that, similar to ex vivo nitric oxide gas exposure, pre-treatment with MAHMA NONOate prevents the pulmonary vasoconstriction and hypertension associated with transfusion of stored packed erythrocytes.

Plasma hemoglobin concentrations, measured 10 to 60 min after commencing transfusion, were greater in sheep transfused with stored packed erythrocytes and MAHMA NONOate-treated stored packed erythrocytes, as compared to sheep transfused with fresh packed erythrocytes (Supplemental Figure 2C). However, treatment with the nitric oxide-donor compound did not further increase the level of circulating cell-free hemoglobin in the plasma of sheep that received MAHMA NONOate-treated, as compared to untreated, stored packed erythrocytes.

Treatment of a stored packed erythrocytes unit with MAHMA NONOate prior to transfusion markedly decreased the ability of circulating plasma to scavenge nitric oxide (Supplemental Figure 2D). Transfusion of fresh packed erythrocytes was not associated with increased plasma nitric oxide-consumption levels. Nitric oxide-scavenging did not differ between fresh packed erythrocytes with or without MAHMA NONOate treatment (Supplemental Figure 2D).

Pre-treatment of the stored packed erythrocytes unit with MAHMA NONOate and subsequent transfusion did not produce systemic vasodilation or alter systemic hemodynamic parameters, including heart rate, mean arterial pressure, systemic vascular resistance index, cardiac index and central venous pressure. Transfusion of a MAHMA NONOate-treated packed erythrocytes unit

also had no effect on arterial or mixed venous blood gas tensions (data not shown).

Taken together, the results show that treatment of a stored packed erythrocytes unit with the short-lived nitric oxide-donor compound MAHMA NONOate prevents transfusion-associated pulmonary vasoconstriction and pulmonary hypertension without causing systemic vasodilation.

Pre-treatment with MAHMA NONOate improves 1 and 24 h erythrocyte survival

The number of MAHMA NONOate-treated, biotin-labeled circulating erythrocytes was measured after transfusion of fresh or stored packed erythrocytes. When fresh packed erythrocytes were pre-treated with MAHMA NONOate, $94.2 \pm 2.3\%$ of the cells were circulating after 1 h (Supplemental Figure 3). After 24 h, $93 \pm 2\%$ of MAHMA NONOate-treated fresh erythrocytes remained in the circulation. In addition, the number of MAHMA NONOate-treated stored erythrocytes which were circulating after 24 h was higher when compared to untreated stored erythrocytes (Supplemental Figure 3). Seven days after transfusion, there was still a difference in the percentage of MAHMA NONOate-treated, compared to control-treated, stored erythrocytes remaining in the circulation.

These findings demonstrate that, similar to NO gas exposure before transfusion, pre-treatment of stored packed erythrocytes with MAHMA NONOate improved the 24 h and 7 day erythrocyte survival.

Pre-treatment of stored packed erythrocytes with MAHMA NONOate increases erythrocyte deformability

To investigate whether the nitric oxide-donor compound increases erythrocyte deformability, the average transit velocity of MAHMA NONOate-treated fresh and stored erythrocytes through the microfluidic cytometer was measured. The average transit velocity of MAHMA NONOate-treated stored erythrocytes was higher than untreated stored erythrocytes (MAHMA NONOate-treated stored erythrocytes vs. control-treated stored erythrocytes, $95 \pm 5 \mu\text{m/s}$ vs. $115 \pm 5 \mu\text{m/s}$, Supplemental Figure 4). These results suggest that, as with exposure to nitric oxide gas, ex vivo exposure of stored packed erythrocytes to a chemical nitric oxide-donor increases erythrocyte deformability.

Supplemental Figure Legends

Supplemental Figure 1:

(A) Cell-free hemoglobin, (B) Nitric oxide-consumption of extracellular hemoglobin, (C) extracellular and (D) intracellular met-hemoglobin percent in both fresh (FRBC) and stored erythrocyte (SRBC) supernatants before and after treatment with 200 μ M MAHMA NONOate. Fresh and stored RBC controls that were exposed to the control gas (90% nitrogen (N₂)/10% oxygen (O₂)) are superimposed (grey dashed lines) as historic controls in panels A-D. All data mean \pm SD. Hb=hemoglobin; RBC=packed erythrocytes; FRBC= fresh packed erythrocytes; SRBC=stored packed erythrocytes; O₂=oxygen; MAHMA=Methylaminehexamethylenemethylamine nonoate.

Supplemental Figure 2:

(A) Mean pulmonary arterial pressure, (B) pulmonary vascular resistance index, (C) plasma hemoglobin levels, and (D) plasma nitric oxide-consumption before, during and after transfusion of both fresh (FRBC) and stored erythrocyte (SRBC) units after treatment with 200 μ M MAHMA NONOate. Fresh and stored RBC controls that were exposed to the control gas (90% nitrogen (N₂)/10% oxygen (O₂)) are superimposed (grey dashed lines) as historic controls in panels A-D. *p<0.01 values of SRBC+10% O₂ differ from FRBC+10% O₂, FRBC+MAHMA NONOate and SRBC+MAHMA NONOate. +p<0.01 values of both SRBC+10% O₂ and SRBC+MAHMA NONOate differ from FRBC+10 % O₂ and FRBC+MAHMA NONOate. All data mean \pm SD. Hb=hemoglobin; RBC=packed

erythrocytes; FRBC= fresh packed erythrocytes; SRBC=stored packed erythrocytes; O₂=oxygen; MAHMA=Methylaminehexamethylenemethylamine nonoate; PAP=mean pulmonary arterial pressure; PVRI=pulmonary vascular resistance index.

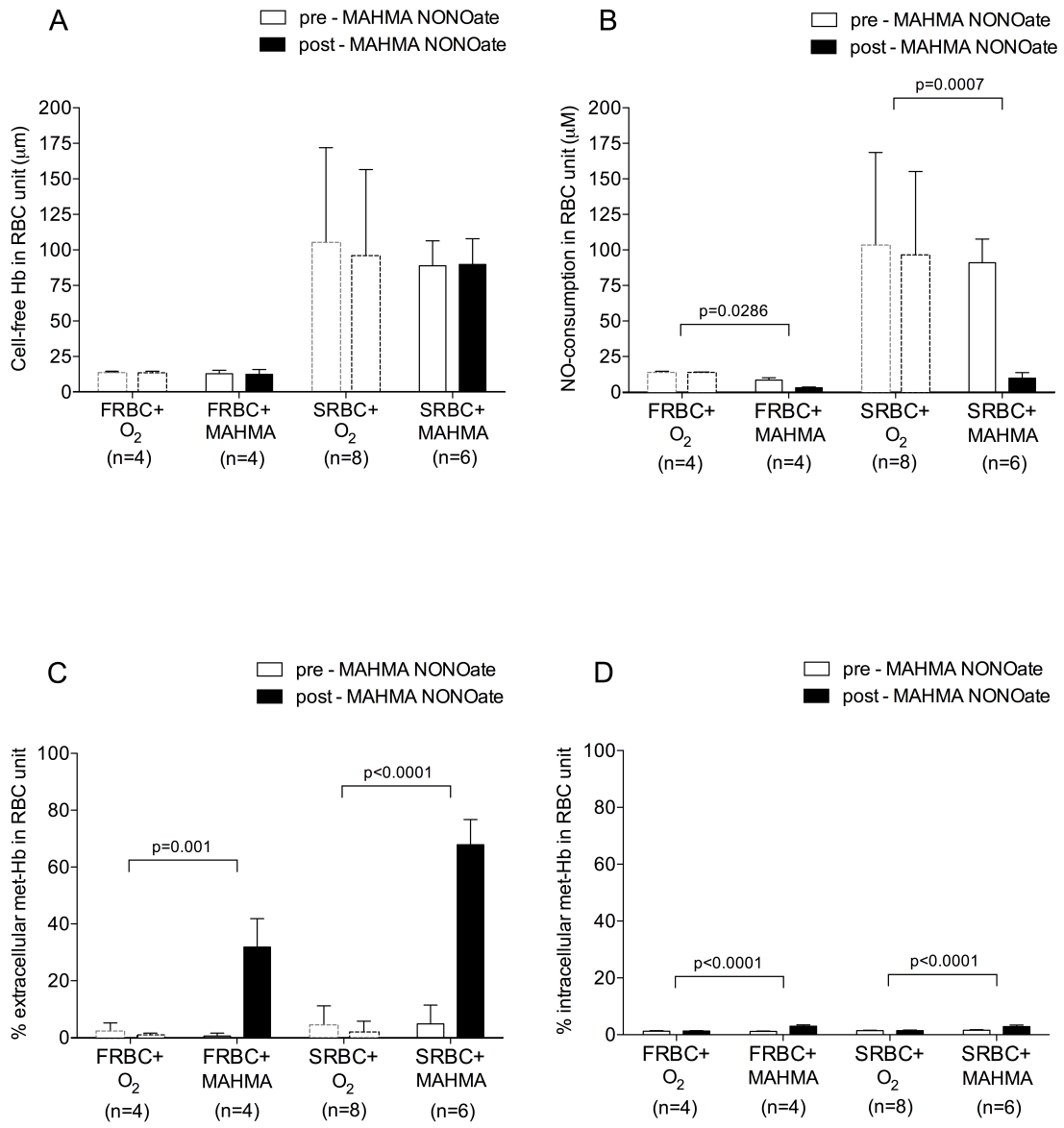
Supplemental Figure 3:

Fresh (FRBC) or stored erythrocytes (SRBC) were treated with 200 μM MAHMA NONOate and the lifespan of circulating biotinylated erythrocytes was measured up to 7 days after transfusion. Fresh and stored RBC controls that were exposed to the control gas (90% nitrogen (N₂)/10% oxygen (O₂)) are superimposed (grey dashed lines) as historic controls. * values of SRBC+MAHMA NONOate differ from SRBC+10% O₂, Bonferroni-adjusted p-values: 60min: p=0.074, 24h: p=0.005, 7 days: p=0.005. All data mean±SD. RBC=packed erythrocytes; FRBC= fresh packed erythrocytes; SRBC=stored packed erythrocytes; O₂=oxygen; MAHMA=Methylaminehexamethylenemethylamine nonoate.

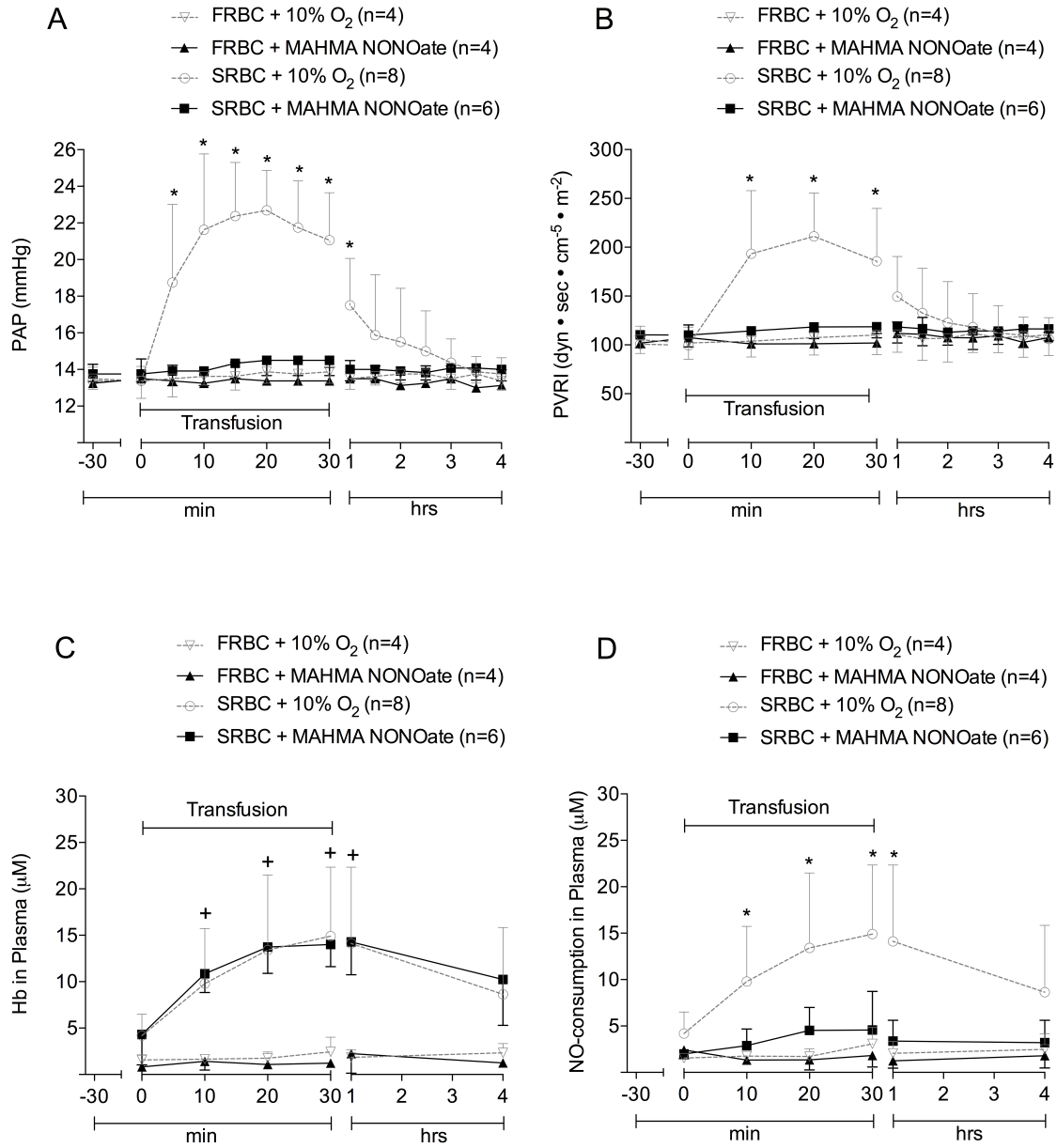
Supplemental Figure 4:

Average velocity to travel across the microfluidic synthetic capillaries of fresh (FRBC) and stored erythrocytes (SRBC) treated with 200 μM MAHMA NONOate. All data mean±SD. RBC=packed erythrocytes; FRBC= fresh packed erythrocytes; SRBC=stored packed erythrocytes; MAHMA=Methylaminehexamethylenemethylamine nonoate.

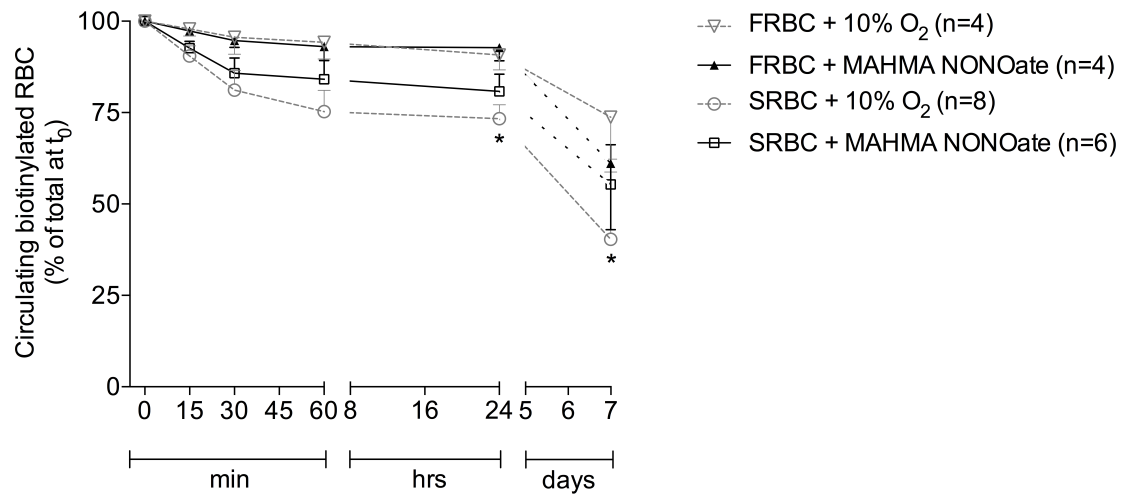
Supplemental Figure 1:



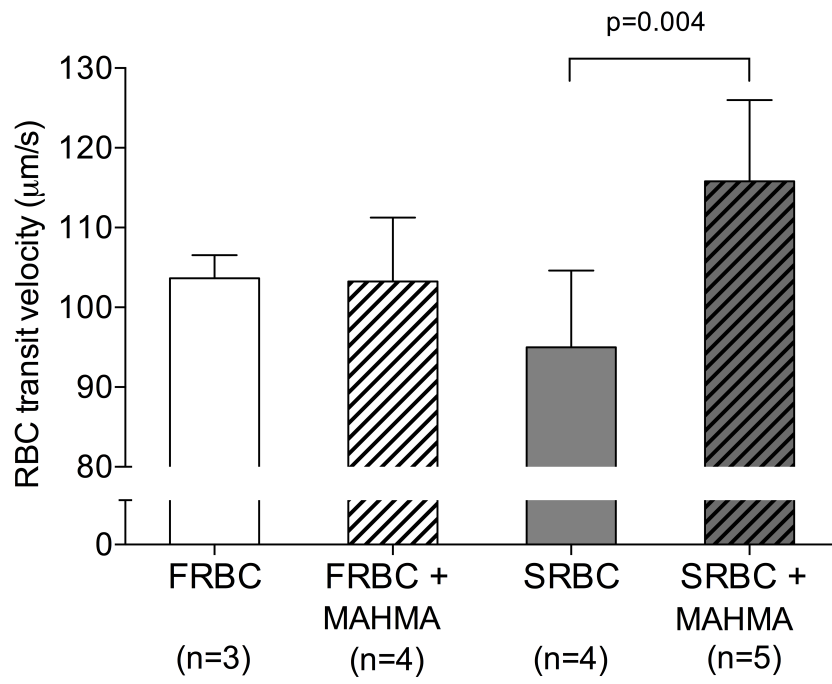
Supplemental Figure 2:



Supplemental Figure 3:



Supplemental Figure 4:



Reference List

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